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Expression of tumor necrosis factor- α converting enzyme (TACE) in endocrine cancers

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ABSTRACT

Tumor necrosis factor- α converting enzyme (TACE) mediates shedding of human epidermal growth factor receptor-4 (HER4). Recent data suggests that released HER4 intracellular domain (4ICD) induces apoptosis in breast cancer. TACE expression, as measured by immunohistochemistry, was observed in 183/383 breast carcinomas, 39/217 ovarian carcinomas, and 16/24 and 17/24 hormone-sensitive and hormone insensitive prostate carcinomas, respectively. HER4 expression was detected in breast carcinomas, using two antibodies recognizing an extracellular or intracellular epitope. TACE expression was predominantly seen in tumors with high levels of 4ICD and membrane HER4. Apoptotic activity was measured by TUNEL assay and cleaved caspase-3 staining in breast carcinomas. There was no significant association between cleaved caspase-3 or TUNEL positivity and 4ICD, whereas TUNEL positivity was seen predominantly in tumors with high levels of internalized HER4. The data presented here show TACE expression in endocrine cancers and further supports a role for TACE in breast cancer

apoptosis.

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INTRODUCTION

Tumor necrosis factor- α Converting Enzyme (TACE) is a zinc-dependent metalloprotein disintegrin membrane-anchored glycoprotein, which belongs to the ADAM protein family (reviewed in 1). This family of proteases is involved in the processing of many cleavable integral membrane proteins, but the physiological substrate for only a few of them has been identified. TACE or ADAM 17 is implicated in inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease 2-4, and was originally identified by its ability to cleave and activate TNF- α precursors 5,6. TACE also plays a central role in protein ectodomain shedding of a variety of structurally and functionally unrelated transmembrane molecules 7, and was recently identified as the proteinase responsible for cleavage of membrane-bound HER4 8. This indicates a central role for TACE also in non-inflammatory conditions such as cancers. A role for TACE in cancer progression was further supported by the finding that TACE can regulate EGFR ligand availability as TACE-mediated activation of proTGF- β was essential for activation of HER1 (EGFR) and thus its downstream signal transduction pathways such as PI3K/Akt or RAS/RAF/MAPK 9,10.

The Human Epidermal growth factor Receptor (HER) family of type-1 receptor tyrosine

kinases consists of four members, EGFR/HER1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4 ¹¹, which regulate cellular proliferation, differentiation and apoptosis in a

growth-factor dependent manner ¹²⁻¹⁴. Aberrant HER signaling is a frequent phenomenon in

various human cancers ¹⁵. Whilst the roles of EGFR and HER2 in cancer progression are

well-characterized, and expression of these oncogenes is linked to poor patient outcome, the

role of HER4 in tumor biology as well as its clinical relevance is still emerging ¹⁶.

Conflicting reports have associated HER4 with adverse prognosis ¹⁷, however, HER4 expression has also been associated with improved patient outcome ¹⁸, reduced tumor cell

proliferation ¹⁹⁻²¹ and good clinicopathological markers such as lower tumor grade in primary, untreated, breast tumors ²². Upon ligand-induced activation, the HER receptors

form receptor homo- and heterodimers and activate, through tyrosine kinase-mediated phosphorylation, downstream intracellular pathways, including the PI3K/Akt and Ras/Raf/MAPK signaling pathway, culminating in cellular responses including proliferation,

differentiation and apoptosis ¹⁵

Recent data suggests that the proteolytically processed intracellular domain of HER4 plays an important role in mediating apoptosis of breast cancer cell lines ^{23,24}. HER4

cleavage within the extracellular domain is initially processed by the proteolytic activity of

TACE ²⁵ and subsequently by the activity of presenilin-dependent γ -secretase ²⁶, which

releases the 80-kDa HER4 intracellular domain (4ICD) into the cytoplasm ²⁷. This intracellular HER4 domain (4ICD) includes the tyrosine kinase domain and is able to translocate to the nucleus ²⁶. During normal breast development and lactation, 4ICD functions as a nuclear chaperone for STAT5A regulating STAT5A transcriptional activation

of the γ -casein promoter ^{14,24}. HER4 over expression has been observed in breast cancers

^{21,28}, and we have recently, using *in vitro* studies, identified 4ICD as a pro-apoptotic BH3-

domain-only protein in breast cancer cell lines, integrating apoptotic signals at the mitochondria and initiating mitochondrial dysfunction ²³. This role of HER4 in mediating

improved patient outcome by affecting the level of apoptosis is not related to activation of

the PI3K/Akt and Ras/Raf/MAPK signaling transduction pathways but requires presenilin-independent

γ -secretase proteolytic processing. Indeed a HER4 variant (HER4-V673I) which lacks a γ -secretase cleavage site was restricted to cytosolic endosome-like structures, failing

to translocate to the mitochondria and induce apoptosis ²⁴. Recently, four HER4 isoforms has

been identified with alternatively splicing within the intracellular cytoplasmic tail (isoform

CYT1 and CYT2) and within the extracellular juxtamembrane region (isoforms JM-a and

JM-b) ²⁹. The two JM-a isoforms (JM-a, CYT1 and JM-a, CYT2) were overexpressed in a

subset of primary breast cancers together with TACE ²⁸ Over expression of JM-a, CYT2

isoform promote HER4 phosphorylation and cancer cell proliferation even in the absence of

ligand stimulation, whereas activation of the other 3 isoforms require ligand stimulation ²⁸.

The expression and function of TACE in cancer development and progression, including endocrine cancers, via its role in HER4-mediated cancer cell apoptosis, has, until

now, been restricted to *in vitro* studies of breast cancer cell lines ^{23,24}. In this study we initially identify TACE expression in breast, prostate and ovary carcinomas and further

investigate if TACE expression is linked to apoptosis in ER-positive breast carcinomas.

MATERIALS AND METHODS

Patients

Breast carcinomas were retrieved from 456 patients diagnosed 1980-1999 and treated with

adjuvant tamoxifen. Estrogen receptor alpha (ER α) expression was confirmed using IHC ³⁰

in 422 cases (92.5%), of which 20 cases (4.4%) were ER α negative. Data presented here

relate only to the 402 ER positive breast carcinomas. Ovarian carcinomas were retrieved

from 220 patients recruited in locally coordinated clinical trials ³¹⁻³⁵. Prostate carcinoma

specimens from 24 patients (24 hormone-sensitive/hormone-insensitive matched pairs; 48

carcinomas in total) were collected retrospectively. Ethical approval was obtained from the

relevant ethics committees for all studies.

Immunohistochemistry (IHC)

IHC was performed on tissue microarrays (TMA; breast and ovary carcinomas) constructed

as previously described ³⁶ or on whole tissue sections (prostate carcinomas) using a standard

immunoperoxidase procedure. The specificity of the TACE antibody was previously verified

2.3. For detection of TACE expression, antigen retrieval was performed by heating sections

overnight at 60°C in 10 mM Tris, 0.25 mM EDTA, pH 9.0. Endogenous peroxidase activity

was quenched with 1% hydrogen peroxide for 20 minutes, and non-specific binding blocked

by casein (Vector Laboratories, CA, USA) for 20 minutes. Primary TACE antibody (C-15,

Santa Cruz biotechnology Inc, CA, USA) was applied overnight at 4°C at a concentration of

0.5 µg/ml, and secondary rabbit anti-goat antibody (Vector Laboratories, CA USA; 1:4000

in casein) was applied for 1 hour at room temperature. EnVision (Dako, Glostrup, Denmark)

was used for signal amplification, and positive staining visualized using 3,3'-

diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, CA, USA). Nuclei were

counterstained with haematoxylin before mounting.

For detection of cleaved (active) caspase-3 expression, antigen retrieval was

performed by microwaving the slides in 1 mM EDTA and 5 mM Tris base, pH 8.0.

Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes,

and non-specific binding prevented by incubation in Serum-Free block (Dako, Glostrup,

Denmark) for 1 hour. Primary antibody detecting cleaved caspase-3 (Abcam, Cambridge,

UK) was applied overnight at 4°C at a concentration of 10 µg/ml. EnVision (Dako, Glostrup,

Denmark), was used for signal amplification and positive staining visualized using 3.3-

diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, CA, USA). Nuclei were counterstained with haematoxylin before mounting.

HER4 expression was evaluated on breast carcinomas using 2 antibodies, H4.77.16, raised against an extracellular epitope, and HFR1, raised against an intracellular epitope,

both from Neomarkers. IHC was performed as previously described ^{30,36}. The specificity of

these two HER4 antibodies, their cellular localization and distribution in this patient cohort

was previously published ³⁶.

TACE and HER4 expression was evaluated using a semi-quantitative weighted histoscore method as previously described ³⁷ whereas the expression of cleaved caspase-3

was measured by counting positive and negative tumor cells. Expression of TACE and

cleaved caspase-3 is shown as percentage of positive tumor cells at any intensity.

Positive

TACE staining is defined as 10% or more TACE positive cells whereas a tumor is regarded

as positive for cleaved caspase-3 if there are any positive cells. HER4 positivity was defined

as previously described ³⁰.

TUNEL assay

In situ TUNEL (terminal deoxynucleotidyl transferase-mediated *in situ* labeling) assay was

performed on breast carcinomas using ApopTag Plus Peroxidase *In Situ* Apoptosis Detection

Kit, Chemicon International) as previously described²³. In brief, devaxed and rehydrated

tissues were proteinase K treated (20 µg/ml) for 15 minutes at room temperature and endogenous peroxidase activity quenched with 3% hydrogen peroxide for 5 minutes.

Reaction buffer containing digoxigenin-labeled dNTPs and terminal deoxynucleotidyl transferase (TdT) enzyme was applied for 1 hour at 37°C, and secondary anti-digoxigenin

conjugate was applied for 30 minutes at room temperature. Apoptosis positive cells were

visualized using peroxidase substrate, and nuclei counterstained using 0.5% methyl green.

The level of apoptosis was evaluated using a 0-400 scale. Intense apoptotic activity was

defined as a score above 200.

Statistical analysis

All statistics were analyzed using SPSS statistical package (version 14.0 for Windows).

Spearman rank tests were conducted to test the associations between expression of TACE,

HER4, cleaved caspase-3 and TUNEL. Kaplan-Meier life tables with log-rank testing were conducted to assess overall and disease free survival of breast cancer patients. A value of $p < 0.05$ was considered statistically significant.

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RESULTS

Clinical and pathological characteristics

All breast cancer patients were treated with tamoxifen for a median of 5 years (range 0.6 –

18 years) and were followed-up for a median of 6.8 years (range 0.11-21.32 years). In addition to tamoxifen, 99/399 (24.8%) patients had chemotherapy (3 unknown) and 110/399

(27.57%) had radiotherapy (3 unknown). There were 74 breast cancer specific deaths and

100 breast cancer relapses, 78 of which occurred during tamoxifen treatment 30. Median time

to relapse for the ovarian cancer patients was 1.02 years (range 0.01- 11.2 years).

Prostate

carcinomas were collected from 24 patients that initially responded to androgen deprivation

(defined by fall in PSA levels of at least 50%), but subsequently relapsed and had tissue preand

post-therapy available for analysis (24 hormone-sensitive/hormone-insensitive matched

pairs; 48 carcinomas in total). Median follow-up time was 4.44 years (range 0.50-7.92 years)

and the median time to biochemical relapse was 2.29 years (range 0.27-6.09 years).

All

patients were either medically (15 patients) or surgically (9 patients) castrated.

TACE expression in endocrine cancers

TACE expression was determined in breast, ovarian and prostate carcinomas (Figure 1-3). In

all three types of endocrine cancers, positive TACE staining was only observed in the cytoplasmic compartments of the invasive tumor, with no staining in normal tissue.

The

staining was often sporadic, reflecting the low percentage of positive cells, however, when

TACE expression was seen it was often very intense.

TACE expression was evaluated in 383/402 breast carcinomas (95%). The remaining 19 cases were excluded from the study because of insufficient tumor material in the cores.

TACE expression was observed in 183/383 carcinomas (47.8%) (Figure 1A and B).

The

median percentage of TACE positive tumor cells was 10 (interquartile range 3-20%;

Figure

1B).

TACE expression was evaluated in 217/220 (98.6%) ovarian carcinomas. The remaining 3 cases were excluded from the study because of insufficient tumor material in the

cores. Positive TACE expression was noted in 39/217 (18%) ovarian carcinomas with a median percentage of TACE positive tumor cells of 2 (interquartile range 0-7%) (Figure 2A and B).

TACE expression was confirmed in 48 prostate carcinomas (24 matched tumors). Positive TACE expression was noted in 16/24 (66.7%) hormone-sensitive tumors and in 17/24 (70.8%) hormone-insensitive tumors (Figure 3A). The median percentage of TACE positive cells was 30 (interquartile range 5-97.5%) in hormone-sensitive tumors compared to 20 (interquartile range 2-90) in hormone-insensitive tumors, ($p = 0.804$; Figure 3B and 3C).

Apoptosis in breast carcinomas

Apoptotic activity was evaluated by both TUNEL assay and staining of cleaved caspase-3.

TUNEL assay was evaluated in 363/402 (90%) breast carcinomas. The remaining 38 cases

were excluded from the study because of insufficient tumor material in the cores.

Apoptotic

activity was detected in 141/363 carcinomas (38.8%), including 21 carcinomas (5.8%) with

intense apoptotic activity defined as a TUNEL score above 200.

The expression of cleaved caspase-3 was evaluated in 359/402 (89%) breast carcinomas

(Figure 4A and B). The remaining 43 cases were excluded from the study because of insufficient tumor material in the cores. Positive staining for cleaved caspase-3, defined as

any staining of tumor cells, was detected in 316/359 carcinomas (88%) (Figure 4B).

Only

few cells in each tumor were stained positive for cleaved caspase-3. The median percentage

of cleaved caspase-3 positive tumor cells (average between the 3 cores) was 1.4% (interquartile range 0.6-2.7%; Figure 4B).

TACE and HER4 and apoptosis in breast carcinomas

Data on HER4 expression in the same patient cohort has previously been reported including

cellular distribution of the two antibodies ^{30,36}. In the present study we correlated TACE

expression and the level of apoptotic activity with the previously used HER4 antibodies,

raised against an extracellular (H4.77.16) or intracellular (HFR-1) epitope of HER4 ³⁶.

A

high level of cytoplasmic TACE was found mainly in tumors with high membrane HER4

expression ($p < 0.001$ for both HER4 antibodies, Spearman rank test, Figure 5), or in tumors

with high levels of cleaved intracellular HER4 (4ICD) ($p=0.016$, HFR-1, Spearman rank test, Figure 5) or full length cytoplasmic HER4 ($p=0.002$, H4.77.16, Spearman rank test, Figure 5). No significant association between TACE expression and nuclear HER4 expression was seen ($p=0.050$ and $p=0.178$ for HFR-1 and H4.77.16, respectively, Spearman rank test, data not shown). No significant association between cleaved caspase-3 and TACE expression or TUNEL and TACE ($p=0.257$ and $p=0.824$, respectively, Spearman rank test, data not shown) was seen.

When measuring apoptotic activity by TUNEL assay, high levels of apoptotic activity was seen in tumors with high levels of full length cytoplasmic HER4 ($p=0.015$, H4.77.16, Spearman rank test, Figure 5), but not in tumors with high levels of 4ICD ($p=0.230$, HFR-1, Spearman rank test, Figure 5). When using cleaved caspase-3 as a measurement for apoptotic activity, no significant association was seen between apoptotic activity and full length cytoplasmic HER4 ($p=0.440$, H4.77.16, Spearman rank test, Figure 5) or 4ICD ($p=0.884$, HFR-1, Spearman rank test, Figure 5). No significant association

between apoptotic activity (TUNEL and cleaved caspase-3) and membrane or nuclear HER4

expression was seen (data not shown).

TACE expression and patient outcome in breast cancer

To analyze the association between TACE expression and patient outcome, patients were

divided into those with positive or negative TACE expression, defined as above and below

10% stained cells at any intensity. Using this definition, no correlation between TACE expression and disease-free ($p=0.877$, log-rank test, data not shown) or overall ($p=0.123$,

log-rank test, data not shown) patient survival were noted.

Apoptosis and patient outcome in breast cancers

To analyze the association between apoptotic activity and patient outcome, patients were

divided into those with or without apoptosis, defined as patients with or without any cleaved

caspase-3 tumor expression or patients with a TUNEL score above or below 200.

Using this

definition, no correlation between apoptotic activity and disease-free or overall survival was

observed (data not shown).

DISCUSSION

TACE was originally identified as the proteinase responsible for ectodomain shedding of

pro-TNF α in inflammatory diseases (reviewed in 1). Recent data has, however, revealed that

upon sequential cleavage by TACE and presenilin-dependent γ -secretase complex, the

intracellular domain of HER4 is released 8,25-27. The association of HER4 expression with

clinical outcome has been suggested to depend on whether HER4 is cleaved (4ICD) or

remains intact on the cell surface. 4ICD, translocated to the nucleus, has been associated

with a worse clinical outcome 38.

We have previously demonstrated *in vitro*, that 4ICD in normal breast development activates and complexes with STAT5A in the cytoplasm to function as a nuclear chaperone

for STAT5A, thus regulating STAT5A-dependent transcriptional activation of the κ -casein

promoter 14,24. We also identified 4ICD in the cytoplasmic compartment as a pro-apoptotic

BH3-only protein in breast cancer cell lines that converts apoptotic signals at the mitochondria, initiating mitochondrial dysfunction and cell death 23. In the current study we

demonstrate TACE expression in tumor cells of breast, ovary and prostate carcinomas. To

further evaluate the *in vivo* role of TACE and HER4 in mediating breast cancer apoptosis,

we have used two HER4 antibodies. The H4.77.16 antibody, raised against an extracellular

fragment, detects predominantly the intact (non-cleaved) membranous HER4 and the HFR-1

antibody, raised against an intracellular epitope, detects intact membranous and the cleaved

cytoplasmic (4ICD) form of HER4. Using this *in vivo* approach we identified a link between

TACE and membrane or cytoplasmic intact HER4 expression as well as between TACE and

cleaved (4ICD) HER4 in breast carcinomas. The association between 4ICD and TACE

expression supports a role for TACE in mediating cleavage of membrane-bound HER4 in

breast cancers and thereby links TACE to the *in vivo* function of 4ICD e.g. apoptosis (Figure

5). This is a support of our previous study showing that proteolytic processing of the cell surface by TACE and presenilin-dependent γ -secretase complex, generating cytosolic 4ICD, is necessary for HER4-mediated apoptosis ²⁴. We have also previously

demonstrated that unprocessed HER4 accumulates in cytosolic endosome-like structures ²⁴.

As TACE has been localized within endosomes ³⁹, the observed correlation between TACE

and cytoplasmic intact HER4 detected by the H4.77.16 antibody could indicate colocalization

of TACE and HER4 in endosomes.

In the present study we measured apoptosis in breast carcinomas using both TUNEL and staining of active (cleaved) caspase-3. Previous studies have shown that TUNEL detects

a markedly higher level of apoptosis than cleaved caspase-3^{40,41}, which may be related to

detection of other forms of cell death i.e. necrosis or detection of DNA breaks that occur

during mitosis or DNA repair. Conversely, non-caspase-3 mediated apoptotic pathways have

also been identified which may suggest that caspase-3 does not detect all apoptotic cells⁴².

Taken together, these data challenge assumptions regarding optimal methodologies for

detection of apoptosis. At present, however, detection of cleaved caspase-3 is regarded as a

more reliable method than the TUNEL assay, despite the fact that caspase-3 may underestimate the level of apoptosis^{40,41}. Using TUNEL assay and detection of cleaved

caspase-3 as two different measurements of apoptosis we were, in the present study, unable

to detect any significant correlation between cleaved HER4 (4ICD) and apoptosis.

Previously, we have, however, shown a link between 4ICD and apoptosis *in vitro* by

measuring chromatin condensation (visual detection of morphological signs of apoptosis

following DAPI staining) in human breast cancer cells transfected with HER4 ²³.

Further

evaluation of the mechanism for HER4 cleavage and the role of cleaved HER4 in mediating

breast tumor cell apoptosis *in vivo*, is clearly required

Activated HER4 has previously been linked to antiproliferative responses ²⁰ and the induction of apoptosis, via TACE/HER4, might therefore be restricted to highly proliferating

tumors. TACE can also activate HER receptor ligands, such as pro-TGF- α , which makes

TACE able to mediate both cancer cell apoptosis and proliferation via EGFR activation. This

could explain the lack of association between TACE and apoptotic activity in this cohort of

ER positive, low risk and poorly proliferating tumors. Instead TACE may mediate tumor cell

progression via activating signal transduction pathways. The lack of association between

TACE expression and apoptotic activity might also be explained by the presence of different

HER4 spliced isoforms which are resistant to TACE cleavage ²⁸.

In addition to breast cancers, we also determined TACE expression in other

endocrine cancers. Positive TACE expression was observed in 39/217 (18%) of ovarian

carcinomas, whilst 16/24 (66.7%) and 17/24 (70.8%) hormone-sensitive and hormone-insensitive

prostate carcinomas, respectively, showed positive TACE expression. Prostate

cancers appear, therefore, to be associated with increased levels of TACE expression, relative to breast and ovarian cancers, but there is no obvious association with hormone

resistance. Presumably, TACE could have a role in tumor progression by activating HER

ligands in these endocrine cancers, as shown previously in breast cancer ⁹, however, to

further investigate if TACE plays a similar role in mediating HER4 ectodomain shedding

and apoptosis in endocrine cancers, larger and well-characterized cohorts of prostate and

ovarian carcinomas need to be analyzed.

In this study we have demonstrated TACE expression in three different endocrine cancers, breast, ovary and prostate. We have also shown that TACE expression in breast

carcinomas correlates with membrane HER4 expression and internalized and cleaved HER4.

HER4 is a pluripotent protein in normal physiology including breast development and lactation as well as in cancers where it is able to modulate both cancer cell apoptosis and cell

proliferation. TACE can activate HER ligands by proteolytic processing and thereby also

plays a role in tumor progression. To further elucidate the role of TACE in endocrine cancers, more information is required. Based on our observations and the current literature

we hypothesize a complex interaction between TACE and HER signaling mediated via the

ability of TACE to cleave HER ligands, promoting cancer progression in some tumors, and

cleavage of HER4, again involving TACE but in this context promoting apoptosis and reducing the aggressive potential of tumors.

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Figure legends

Figure 1

(A) Photomicrographs of IHC staining in breast carcinoma showing only cytoplasmic TACE

protein expression. Original magnification: x 400. (B) Histogram showing the number of

breast cancer patients and the percentage of TACE positive cancer cells.

Figure 2

(A) Photomicrographs of IHC staining in ovary carcinoma showing only cytoplasmic TACE

protein expression. Original magnification: x 400. (B) Histogram showing the number of

ovary cancer patients and the percentage of TACE positive cancer cells.

Figure 3

(A) Photomicrographs of IHC staining in prostate carcinoma showing only cytoplasmic

TACE protein expression. Original magnification: x 400. (B) Histogram showing the number of hormone sensitive patients and the percentage of TACE positive cancer cells. (C)

Histogram showing the number of hormone insensitive patients and the percentage of TACE positive cancer cells.

Figure 4

(A) Photomicrographs of cleaved caspase-3 (apoptotic activity) in breast carcinoma.

Original

magnification: x 400. (B) Histogram showing the number of breast cancer patients and the percentage of cells stained positive for cleaved caspase-3.

Figure 5